Lung Vascular Remodeling, Cardiac Hypertrophy and Inflammatory Cytokines in SHIV\textit{nef}-infected macaques

Sharilyn Almodovar\textsuperscript{1,2,*}, Jessica Swanson\textsuperscript{1}, Luis D. Giavedoni\textsuperscript{3}, Sreetharan Kanthaswamy\textsuperscript{4} Carlin S. Long\textsuperscript{5}, Norbert F. Voelkel\textsuperscript{6}, Michael G. Edwards\textsuperscript{1}, Joy M. Folkvord\textsuperscript{7}, Elizabeth Connick\textsuperscript{7}, Susan V. Westmoreland\textsuperscript{8}, Paul A. Luciw\textsuperscript{9}, Sonia C. Flores\textsuperscript{1}

Affiliations:
\textsuperscript{1}Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado.
\textsuperscript{2}Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, Texas.
\textsuperscript{3}Dept. of Virology and Immunology, and Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas.
\textsuperscript{4}School of Mathematical and Natural Sciences, New College of Interdisciplinary Arts and Sciences, Arizona State University, Arizona.
\textsuperscript{5}Department of Medicine, University of California, San Francisco, San Francisco, California.
\textsuperscript{6}Victoria Johnson Center for Obstructive Lung Diseases, Virginia Commonwealth University, Richmond, Virginia.
\textsuperscript{7}Division of Infectious Diseases, Department of Medicine, University of Arizona College of Medicine, Tucson, Arizona.
\textsuperscript{8} (former) New England Primate Research Center, Division of Comparative Pathology, Southborough, Massachusetts.
\textsuperscript{9}Center for Comparative Medicine, University of California, Davis, California.

*Corresponding author
Sharilyn Almodovar, PhD.
Texas Tech University Health Sciences Center
Department of Immunology and Molecular Microbiology
Telephone: (806) 743-1091 | Fax: (806) 743-2334
Email: sharilyn.almodovar@ttuhsc.edu
Abstract

Fatal pulmonary arterial hypertension (PAH) affects HIV-infected individuals at significantly higher frequencies. We previously showed plexiform-like lesions characterized by recanalized lumenal obliteration, intimal disruption, medial hypertrophy, and thrombosis consistent with PAH in rhesus macaques infected with chimeric SHIV\textit{nef} but not with the parental SIVmac239, suggesting that Nef is implicated in the pathophysiology of HIV-PAH. However, the current literature on non-human primates as animal models for SIV(HIV)-associated pulmonary disease reports the ultimate pathogenic pulmonary outcomes of the research efforts however, the variability and features in the actual disease progression remain poorly described, particularly when using different viral sources for infection. We analyzed lung histopathology, performed immunophenotyping of cells in plexogenic lesions pathognomonic of PAH and measured cardiac hypertrophy biomarkers and cytokine expression in plasma and lung of juvenile SHIV\textit{nef}-infected macaques. Here we report significant hematopathologies, changes in cardiac biomarkers consistent with ventricular hypertrophy, significantly increased levels of interleukin-12 and GM-CSF and significantly decreased sCD40L, CCL-2 and CXCL-1 in plasma of the SHIV\textit{nef} group. Pathway analysis of inflammatory gene expression predicted activation of NF-κB transcription factor RelB and inhibition of bone morphogenetic protein type-2 in the setting of SHIV\textit{nef} infection. Our findings highlight the utility of SHIV\textit{nef}-infected macaques as suitable models of HIV-associated pulmonary vascular remodeling as pathogenetic changes are concordant with features of idiopathic, familial, scleroderma and HIV-PAH.

Keywords

\textit{SHIVnef, pathogenesis, pulmonary vascular remodeling, inflammation.}
Introduction

With the advent of antiretroviral therapy, human immunodeficiency virus (HIV) infection is now a manageable chronic disease. Nonetheless, HIV-infected individuals face long-term complications including pulmonary arterial hypertension (PAH). Life-threatening PAH is relatively rare (10-50 individuals per million) but afflicts HIV-infected individuals at significantly higher frequencies (1 in 200) regardless of antiretroviral drug status, HIV viral load, CD4 counts or duration of HIV infection. HIV-associated PAH (HIV-PAH) is clinically indistinguishable from other forms of PAH, and is characterized by high pulmonary artery pressures, inflammation, pulmonary vascular remodeling, increased circulating inflammatory cytokines and the presence of cells that obliterate the lumina of pulmonary arteries and form plexiform lesions. There is no definitive proof that HIV directly causes PAH or infects pulmonary endothelial cells. Nevertheless, HIV proteins play key roles in PAH-associated pulmonary vascular remodeling because their interactions with molecular partners in the infected cells induce inflammation, oxidative stress and deregulate apoptosis and proliferation of vascular endothelial cells.

Non-human primates infected with simian immunodeficiency virus (SIV) recapitulate many aspects of HIV pathogenesis and end organ diseases. Chalifoux et al. reported intimal and medial thickening in pulmonary arteries, and pulmonary thrombosis with recanalization in rhesus macaques infected with SIVmac251 strain. George et al. reported similar pulmonary vascular changes in SHIVenv89.6P-infected cynomolgus macaques and SIVΔB670-infected rhesus macaques, including occasional plexiform lesions characterized mostly by collagen deposition and significantly elevated
right atrial, right ventricular systolic, and pulmonary artery pressures (14, 15).

Pulmonary vascular remodeling consistent with PAH was also reported in morphine-treated rhesus macaques infected with SIVmacR71/17E (44). In addition, pulmonary arterial occlusive thrombi were reported in two macaques infected with an engineered microglia-tropic SHIV-B0159N4-p2 at 73 weeks post-infection (WPI) (10). We previously showed plexiform-like lesions characterized by recanalized luminal obliteration, intimal disruption, medial hypertrophy, and thrombosis consistent with PAH in rhesus macaques infected with chimeric virions expressing human HIV nef in an SIVmac239 genetic backbone (SHIV nef), but not in macaques infected with the parental SIVmac239. The SIV-infected animals exhibited lung pathology without pulmonary vascular lesions. (33, 34). In addition, we reported that HIV Nef protein co-localizes with endothelial cells and mediates Golgi fragmentation in PAH-like plexiform lesions in macaques infected with SHIV nef, suggesting a direct pathogenetic relationship (31, 33, 34, 41). We translated the animal studies to humans and found Nef signature sequences associated with PAH (2). Altogether, these results led us to propose a role for Nef in the development of HIV-PAH.

The current literature portraits non-human primates as animal models for SIV(HIV)-associated pulmonary disease by reporting the ultimate pathogenic pulmonary outcomes of the research efforts however, the features in the actual disease progression remain poorly described. Therefore, we sought to gain further insights into inflammatory marker production and vascular remodeling in rhesus macaques in the context of SHIV nef infection. Herein, we show the spectrum of mild to aggressive pulmonary vascular disease in SHIV nef-infected macaques. Furthermore, we examined
whether cytokines previously implicated in PAH were present in this animal model. We found that macaques infected with SHIV\textit{nef} resulted in development of pulmonary vascular lesions and expressed a gene program characteristic of pathologic cardiac hypertrophy frequently seen in hearts of patients with PAH. Furthermore, these studies demonstrated alterations in cytokine expression consistent with other forms of PAH, highlighting the long-standing utility of this model to dissect pathogenic mechanisms.

**Materials and Methods**

**Inoculation and care of rhesus macaques.** Juvenile (3-year old) male rhesus macaques (\textit{Macaca mulatta}, Mmu) were purchased from a D retrovirus-free and SIV-free colony at the California Regional Primate Research Center (CNPRC, n= 6) and the New England Primate Research Center (NEPRC, n= 6); we used two different centers due to contractual reasons. Parentage and ancestry analyses were performed as described by Kanthaswamy et al. (26). All animals at NEPRC and CNPRC were pure Indian rhesus, except animal CNPRC 6, which was confirmed as a Chinese-Indian hybrid. Animals were inoculated intravenously with 500 ul of cell-free SHIV\textit{nef}\textsubscript{SF33A} (32). Figure 1 depicts the natural history of the SHIV\textit{nef} virions used for infection. The chimeric SHIV\textit{nef} SF\textsubscript{33A} used to infect our NEPRC monkeys was passaged once in vivo, upon recovery from a macaque that died of simian AIDS at 53 weeks post-infection (32). On the other hand, the SF33\textsubscript{A2} virus used to infect the CNPRC group was passaged twice. All animals remained antiretroviral drug-naïve throughout this study. The animals were cared for in accordance with the standards of the American Association for Assessment and Accreditation of Laboratory Animal Care. Animals were monitored daily for
hydration, appetite, weight loss, temperature, diarrhea, behavioral changes, activity level, and opportunistic infections and euthanized by intravenous overdose of sodium pentobarbital when signs of immunodeficiency, i.e. 15% weight loss in two weeks, ~7% dehydration, persistent leukopenia, presence of opportunistic infections or abdominal lesions were detected.

**Histopathology.** Tissues collected at necropsy were formalin-fixed and paraffin-embedded or flash frozen in OCT. Lung serial sections lungs were stained with hematoxylin and eosin, as per standard protocols, scanned digitally using the Aperio ScanScope software (Vista, CA) and examined by two experienced pathologists in a blinded fashion. For analyses using immunofluorescence microscopy, formalin-fixed paraffin-embedded serial lung sections were stained with fluorescently labeled antibodies as follows: Nef (mouse monoclonal antibody; Advanced BioTech, Eldersburg, MD), biotinylated goat anti-mouse and streptavidin-Texas Red antibody (Molecular Probes, Frederic, MD). Sections were washed and incubated with 10% donkey serum and stained for lymphocytes (rabbit anti-CD3; Dako), stem cells (rabbit anti-CD34; Abcam, Cambridge, MA), macrophages (rabbit anti-CD163; Sigma, St. Louis, MO), or smooth muscle (rabbit anti-smooth muscle actin; Abcam). After incubation with primary antibodies, sections were stained with anti-rabbit AF647 (Molecular Probes). In addition, endothelial cells were labeled with von Willebrand factor antibody conjugated to FITC (GeneTex, Irvine, CA). Coverslips were mounted using VECTASHIELD HardSet mounting media with DAPI (Vector Labs, Burlingame, CA). Isotype controls were also included. The pixel intensities were measured in grayscale
images and a threshold was set for all images using a common factor of the mean pixel intensity. The threshold was used to calculate mean fluorescence intensities using Image J processing program; intensities from 3 images captured for each macaque were averaged and plotted.

Cardiac ventricle gene expression. Total RNA from the left or right ventricles was individually isolated from four SHIV nef-infected macaques and one SIV-infected control with TRIzol reagent (Life Technologies) according to manufacturer's specifications. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase and random hexamer primers as described previously (5, 24). Expression of cardiac hypertrophy genes was quantified by real-time PCR.

Laboratory assessments and course of infection. Blood was drawn from monkeys anesthetized with an intramuscular injection of ketamine hydrochloride at various time points (5 mL peripheral blood) for routine SIV viral load testing (measured by real time TaqMan PCR, with a sensitivity of ~50 copies/ml), complete blood counts (CBC) and peripheral blood lymphocyte subset counts by flow cytometry. Values were compared to normal reference values (7, 17); median values relative to reference intervals were used to define hematopathologies. We measured cytokine and chemokine levels in plasma using Luminex multiplex microbead immunoassay arrays, as described (16, 20). The Luminex 20-plex bead combination included in-house reagents and commercial beads purchased from Life Technologies (Frederic, MD), EMD Millipore (Billerica, MA), and
R&D Systems (Minneapolis, MN). All animals remained antiretroviral drug-naïve throughout this study.

*Inflammatory gene expression profiles.* Cell-associated total RNA was extracted from flash-frozen lung tissues with Qiagen miRNeasy Mini Kit. The quality of the extracted RNA was assessed in a R6K ScreenTape (Agilent Technologies, Santa Clara, CA). The cDNA was synthesized using the RT² First Strand Kit; reactions for quantitative PCR were prepared with the RT² SYBR Green Fluor qPCR Master Mix Kit (SABiosciences, Valencia, CA). Samples were amplified using the PCR targeted array for Rhesus Macaque Inflammatory Cytokines & Receptors as per manufacturer protocols. The housekeeping genes beta-2-microglobulin and hypoxanthine-guanine phosphoribosyltransferase-like were used for normalization. Gene expression was determined via the ΔΔCt method; fold changes were calculated based on difference in gene expression between SHIV nef- and SIVmac239-infected macaques. Gene expression results were queried for potential biological relevance using the Ingenuity Pathway Analysis (IPA) software (Qiagen, www.ingenuity.com).

*Bioinformatics Analysis.* The fold change data for the 84 genes compared in the SHIV nef vs. SIVmac239 infections were uploaded into Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) and used to predict potential pathways affecting these genes. IPA uses a Fisher’s exact test to identify over-represented connected biological units in a defined set of genes, which can include pathways, cellular functions, or known targets of regulatory genes. In some cases, a confidence score, or z-score, can be made on the
activation state of the pathway or upstream regulator based on the expression pattern of the associated genes (28). The activation z-score is used to infer likely activation states of upstream regulators based on comparison with a model that assigns random regulation directions; regulators with higher z-scores have more evidence of activation, while regulators with lower z-scores have more evidence of inhibition over the random model.

Results

*SHIV*<sup>nef</sup>-infected rhesus macaques show variable infection dynamics after passage in vivo.*

In this study, animals at NEPRC were infected with chimeric SHIV<sub>nefSF33</sub> after one passage *in vivo*, while the CNPRC monkeys were infected with SHIV<sub>nefSF33</sub> after two serial passages, as indicated in Figure 1A. Baseline and longitudinal measurements of plasma SIV RNA confirmed productive infection in all infected animals. Peak plasma viral load during acute infection was observed at two weeks post-inoculation. Macaques in the CNPRC cohort trended to have higher viral loads during the acute phase (mean, 1.24x10<sup>7</sup> copies/ml) compared to animals at NEPRC (mean, 3.05x10<sup>6</sup> copies/ml, p=0.0952) (Figure 1B). Survival times post-infection in the CNPRC group ranged from 12 to 62 weeks, with median 30.5 weeks and mean 33 weeks (95% CI: 11.7, 54.3). In the NEPRC group, the survival times ranged from 12 to 80 weeks, with median 21.5 weeks and mean 34 weeks (95% CI: -16.3, 83.8). Differences in survival between the cohorts were not statistically significant different (p = 0.7679, Log-rank Mantel-Cox test).
Pulmonary vascular remodeling in SHIVnef-infected macaques.

SHIVnef-infected monkeys in the NEPRC cohort showed mild adventitial pulmonary vascular thickening with inflammatory infiltrates (see representative images in Figure 2A). In contrast, the CNPRC animals (Figure 2B) showed significant pulmonary vascular remodeling. Specifically, pathological remarks in the first animal, CNPRC 1, included predominant bronchial associated lymphoid tissue (BALT), medial hypertrophy, prominent lymphocytic infiltration, and vascular remodeling at 12 weeks post-infection (WPI). Macaque CNPRC 2 and 3 showed apoptotic bodies, presence of neutrophils, pneumonia, giant cells indicative of severe infection and foci of lymphocyte infiltrations at 13 and 24 WPI. Macaque CNPRC 4 showed major remodeling, obstructive recanalized lesions, plump endothelial cells, intravascular thrombotic bodies, medial hypertrophy, and plexiform lesions at 37 WPI; pentachrome staining identified fibrin and muscle within the lesions (Figure 3). HIV Nef protein was detected in the lesions of this animal by immunohistochemistry (Figure 4). No vascular lesions were found in the spleen, lymph nodes, kidney, liver, and colon, suggesting pulmonary artery-specific pathology (Figure 5). Despite the severe pulmonary vascular remodeling observed in animal CNPRC4, very little inflammatory cell infiltration was seen in the lungs compared to its mates. CNPRC 5 showed prominent alveolar proteinosis, which made it difficult to identify lung abnormalities. Lastly CNPRC 6 showed prominent lymphoid follicles indicative of exuberant bronchiolitis at 62 WPI (Figure 2B). Because aging is known to promote remodeling of the pulmonary vasculature, with an increase in muscle content of the pulmonary artery and stiffness (45), we examined archival material consisting of H&E-stained lung sections of two adult rhesus macaques, aged 11 and 14 years old.
infected with the parental SIVmac239 for 78-81 weeks and designated as old, long-term non-progressors. Despite the advanced age of the SIVmac239-infected monkeys compared to our juvenile SHIV nef-infected macaques, the former exhibited adventitial pathology with inflammation without vascular lesions (Figure 2C).

**Immunophenotypic characterization of cells in the lungs of SHIV nef-infected macaques.**

Given the evidence of pulmonary vascular remodeling in the SHIV nef-infected macaques from the CNPRC group, we characterized the pulmonary vascular cells. Lung tissue sections were stained with fluorescent-labeled antibodies to T lymphocytes (CD3), macrophages (CD163), smooth muscle actin, hematopoietic stem cells (CD34), or proliferating cells (Ki-67) multiplexed with markers for endothelial cells (von-Willebrand factor), Nef, and nuclei (DAPI). Notably, there were numerous Ki-67 positive cells in the pulmonary plexogenic lesion in animal CNPRC 4 (see representative images in Figure 6). Quantification of immunofluorescent signals for cellular markers in each animal (Figure 7) shows that macaque CNPRC 1 (12 WPI) had the highest CD3 (p=0.0259) and CNPRC 2 (13 WPI) had the highest CD163 levels (p=0.0116) compared to their mates. The macaque with exuberant pulmonary vascular remodeling at 37 WPI (CNPRC 4) had the highest smooth muscle actin (p=0.0199), CD34 (p=0.0431), and Nef (p=0.0116). There was no significant difference in signals for Ki-67 or Factor VIII among the CNPRC macaques. Because *in situ* thrombosis plays a pathophysiologic role in PAH, we analyzed the platelet marker CD61 in the pulmonary vasculature. Immunohistochemical analyses showed higher intravascular CD61 staining in animal CNPRC 4 (Figure 8), suggesting intrapulmonary platelet aggregation.
**Cardiac hypertrophy markers.**

We measured cardiac hypertrophic gene expression in four CNPRC macaques compared to one SIVmac239-infected control. Cardiac mRNA gene expression changes associated with pathological hypertrophy include changes in the relative expression of the α and β myosin heavy chain (MHC) isoforms where α is repressed, decreased expression of sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA), and increased expression of atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP). Consistent with the generalized induction of pathologic cardiac hypertrophy, we found that expression of the natriuretic peptide BNP was increased in ventricles of all CNPRC animals examined while ANP was up-regulated in LVs of all experimental animals but in the RV of only CNPRC4. α-MHC RNA was repressed in all CNPRC samples with the exception of the RV of CNPRC4. Beta Adrenergic gene expression also showed a generalized pattern of repression in CNPRC animals when compared with controls with the exception of CNPRC4 where the Beta 2 AR expression was increased. The relative abundance of SERCA was also decreased in all of the SHIV nef macaques relative to the control. These results are shown in Figure 9.

**Hematopathologies in SHIV nef-infected macaques in the CNPRC cohort.**

Complete blood counts (CBC) collected longitudinally in all macaques were compared to reference CBC values for male rhesus macaques (7, 17). For most endpoints, the median CBC values remained within normal levels in the NEPRC macaques throughout the course of the study. We found persistent lymphopenia (defined as low lymphocyte counts relative to reference values) in the animal euthanized at 27 WPI
(NEPRC4). On the other hand, several blood cell abnormalities were found in the CNPRC animals. Specifically, most of the CNPRC monkeys (CNPRC 2,3, and 4) had significantly low hemoglobin and hematocrit values compared to their SHIV\textit{nef}-infected mates, suggesting anemia. The animal euthanized at 37 WPI (CNPRC 4) also had significant neutrophilia (median 10230 cells/ul, 95%CI: 7826,18055), with persistent lymphopenia (median 1915 cells/ul blood, 95%CI: 1137,3101) and a striking thrombocytosis (high number of platelets (median 4.58x10^5 cells/µl, 95%CI: 3.25x10^5, 5.31x10^5) compared to the other SHIV\textit{nef}-infected macaques at CNPRC. These hematopathologic findings are shown in Table 1 and in Figure 10.

Furthermore, we compared the CBC values between the two cohorts (excluding uninfected controls) and found that the SHIV\textit{nef}-infected monkeys in the NEPRC were significantly more lymphocytopenic than their CNPRC counterparts (p=0.0046, Mann Whitney Test). In addition, animals in the CNPRC group had significantly lower RBC, hemoglobin, and hematocrit values than the NEPRC animals (p<0.0001, Student’s T test). No statistically significant differences were observed in white blood cell counts or platelets between the two cohorts (Figure 11 and Table 2). Altogether, these data suggest a worse course of disease in the CNPRC animals infected with chimeric SHIV\textit{nef} passaged twice than the NEPRC monkeys infected with SHIV\textit{nef} virus passaged only once.

\textit{Inflammatory biomarkers in plasma and lungs of SHIV\textit{nef}-infected rhesus.}

Plasma cytokines were measured in six CNPRC SHIV\textit{nef} and four SIV\textit{mac239}-infected rhesus. Group-specific intercepts and slopes for weeks post-infection were
estimated from a linear mixed-effects model for each of the tested cytokines (Table 3). Compared to the SIV group, the average cytokine value was significantly higher in the SHIV<nf> group for cytokines IL-12 (p=0.0002) and granulocyte macrophage colony stimulating factor-2 (GM-CSF, p<0.0001), and significantly lower in the SHIV<nf> group for soluble CD40 ligand (sCD40L, p=0.0002), monocyte chemotactic protein-1 (CCL-2/MCP-1, p=0.0063) and growth-related oncogene alpha (CXCL-1/GRO-a, p=0.0038, Figure 12). The relationship between time and cytokine was not different between the two groups for any of the inflammatory cytokines.

We next analyzed inflammatory gene expression in the lungs of the six CNPRC SHIV<nf>-infected macaques compared to two SIVmac239-infected rhesus macaques by PCR array. We found that the SHIV<nf>-infected macaques had ~6-fold decrease in CD40L and a ~3-fold increase in interleukin 6, a 4-fold increased CCL2/MCP-1 and CCL20/MIP3a, and a ~12-fold increase in CCL23 (Table 4). All the gene expression data were analyzed using the Ingenuity Pathway Analysis software to identify potential transcription effectors in the context of SHIV<nf> infection. Relative to SIVmac239, we found the regulators bone morphogenetic protein-2 (BMP-2) and the transcription factor RelB were predicted to be the most inhibited and activated with SHIV<nf> infection, respectively, based on the direction of expression their target genes (Figure 13).

Discussion

Herein we present the first comprehensive examination of pulmonary vascular changes and associated biomarker alterations in 2 separate cohorts of SHIV<nf>-infected macaques. This study confirms and extends our previous work demonstrating
pulmonary vascular lesions consistent with HIV-PAH in rhesus macaques infected with SHIV\textit{nef}, and that these lesions are not seen in animals infected with parental SIV\textit{mac}239.

An observation derived from these studies is that the CNPRC animals infected with SHIV\textit{nef} virions serially passaged twice had higher viral loads during the acute phase of infection, lower viral set points, faster progression to simian AIDS and worse pulmonary vascular phenotypes than the animals infected with virus passaged only once. Bioinformatics sequence analyses are underway to elucidate whether virus passaging introduced mutations that translated to severe pulmonary disease in macaques. We further demonstrate that SHIV\textit{nef} macaques exhibit cardiac hypertrophy and changes in plasma and lung cytokines consistent with PAH. Collectively, these data suggest that the SHIV\textit{nef} rhesus macaque model recapitulates many aspects of HIV-PAH and its usefulness to further dissect the mechanisms that promote pulmonary vascular remodeling.

We noted vascular remodeling in the pulmonary vasculature but not in extra-pulmonary tissues. Consistent with our previous studies, we detected Nef within the plexiform lesions but the presence and/or abundance of Nef in non-pulmonary tissues and whether there is a correlation with presence/absence of vascular remodeling remains to be elucidated. We also noted differences in lung histopathology between the two cohorts of SHIV\textit{nef} infected rhesus macaques. The characteristic histopathology of PAH includes excessive proliferation of smooth muscle cells and endothelial cells to the point of lumen obliteration, with perivascular inflammatory cell infiltrates and intravascular thrombosis (1, 3, 39, 46). While the NEPRC cohort showed mostly
adventitial pulmonary vascular changes, the SHIV\textit{nef}-infected macaques at CNPRC featured severe pulmonary artery-specific remodeling, including perivascular inflammatory cell infiltrates, medial hypertrophy, and obstructive plexiform lesions, and intravascular thrombosis particularly in macaque CNPRC 4. Histological and immunophenotypic characterization of cells in the lungs of SHIV\textit{nef}-infected monkeys as a function of time suggest that once the plexiform lesions form, there is no longer evidence of inflammation. Numerous proliferating cells were observed in the pulmonary plexogenic lesion in animal CNPRC 4, which aligns with early descriptions of HIV-PAH lesions (8). Consistent with our previous studies (33, 34), our findings were exclusive to SHIV\textit{nef}-infected macaques but not in SIVmac\textsubscript{239}-infected monkeys, even after longer duration of SIV infection and advanced age. Based on pentachrome staining, it is noteworthy that the obliterative lesions in our SHIV\textit{nef} model are rich in fibrin and muscle cells, while the lesions reported in other SIV models are characterized by collagen deposition (6, 15, 44). Therefore, the mechanisms at play in the formation of PAH-associated lesions in the SHIV\textit{nef} and SIV models are most likely different.

Moreover, although the SIV and SHIV models have contributed to the overall knowledge to the field of HIV-PAH, the fact that the various studies used different macaque species infected with different SIV/SHIV strains complicated by the multifactorial nature of pulmonary vascular remodeling and PAH may confound some of the conclusions.

Our animals remained antiretroviral drug naïve throughout the course of the study and were humanely euthanized when signs of immunodeficiency were detected. Similar to human HIV disease, our macaques progressed to simian AIDS at different rates and therefore, were terminated between 12 and 62 WPI. This represents an
inherent limitation in our study because the broad range in length of viral infection makes it difficult to compare vascular pathologies. Our studies were also limited by the lack of hemodynamic data for a conclusive diagnosis of PAH in the setting of SHIV\textit{nef} infection. While increased pulmonary artery pressures have been already reported in infected macaques as early as 12 weeks post-infection (15), our histological and cardiac hypertrophy marker lines of evidence support the observation of pulmonary vascular remodeling with right ventricular hypertrophy. There was a generalized change in the expression of a number of cardiac-myocyte selective genes consistent with what has been described as the pathologic gene program (38, 47) in the CNPRC animals examined. Specifically, there was an increase in expression of the natriuretic peptides, a repression of SERCA and Beta Adrenergic Receptor subtypes and a change in MHC isoforms favoring the $\beta$- over the $\alpha$-isoform. Several blood cell abnormalities were also found in the CNPRC animals, including significant neutrophilia, anemia, and lymphopenia. Of note, macaque CNPRC 4 showed severe anemia with thrombocytosis with intrapulmonary thrombosis. Interestingly, small vessel thrombosis is a common finding in idiopathic PAH (3, 4). Whether intravascular pulmonary thrombi developed in situ or by embolization remains an open area of investigation.

We found significantly increased levels of the pro-inflammatory molecules interleukin-12 and GM-CSF in plasma of the SHIV\textit{nef} group. It is known that IL-12 is increased in the serum of patients with scleroderma-associated PAH (30). It is also known that GM-CSF increases inflammatory cell recruitment and exacerbates PAH when the expression of bone morphogenetic receptor type-2 (BMPR-2) is reduced (40). In line with this, bioinformatic predictions based on inflammatory expression in the lungs
of our SHIV\textit{nef}-infected macaques and curated databases pointed to inhibition of bone morphogenetic protein type-2 (BMP-2) as a likely involved pathway. BMP-2 is a negative regulator of smooth muscle cell growth associated with familial, idiopathic and anorexigen-associated PAH (13, 22, 35). HIV Tat and cocaine also exacerbate BMPR downregulation and increase proliferation of pulmonary vascular smooth muscle cells (11). In addition, the bioinformatics models predicted, with high confidence, activation of the non-canonical NF-κB transcription factor RelB. RelB is a marker of exacerbations in patients with chronic obstructive pulmonary disease (29) and exerts anti-inflammatory effects in smoke-induced pulmonary acute inflammation in mice (49). Tat-transgenic mice exhibit increased RelB in the brain, where RelB may counter-regulate the pathogenic outcomes of HIV Tat (27). Whether the predicted increase in RelB in the lungs of SHIV\textit{nef} macaques reflects the lung responses to Nef-induced inflammation remains an intriguing question. Taken together, the gene expression results in the lungs of SHIV\textit{-nef} infected macaques are consistent with findings in idiopathic, familial and HIV-PAH.

We also found significantly lower sCD40L, CCL2 and CXCL-1 in plasma of SHIV\textit{nef}-infected macaques; sCD40L was \~6 times decreased in the lungs of SHIV\textit{nef} macaques. Dysregulation of sCD40L is known to impact immunoglobulin class switching (19) and vascular disease (36). In addition, Nef is known to highjack CD40-dependent immunoglobulin class switching pathways in B cells (37). Hence, our finding of decreased sCD40L in plasma and lungs of SHIV\textit{nef}-infected macaques compared to SIV-infected monkeys supports potential Nef-mediated immune dysfunction that is evident in the SHIV\textit{nef} model. Surprisingly, we also found significantly decreased CCL-
2 in the plasma but ~4X-fold increase in the lungs of SHIV nef-infected macaques. CCL-2 is crucial for Th1 immune responses recruitment of macrophages to inflamed tissues (21). Despite the apparent discordance between CCL-2 levels in the periphery and the lung, it is conceivable that CCL-2 redirects the migration of monocytes/macrophages into the lungs in the context of SHIV nef infection. Our future studies will measure the levels of the neutrophil recruiter CXCL-1 in the lungs of SHIV nef-infected monkeys in order to relate CXCL-1 levels in the plasma and lungs. Lastly, our finding of increased CCL23 and IL-6 in the lungs of SHIV nef-infected monkeys is consistent with PAH associated with systemic sclerosis (48), and idiopathic and familial PAH(23, 43), respectively.

Here we demonstrate the pathobiology of SHIV nef infection in rhesus macaques and present compelling evidence that further highlights the Nef-mediated role in PAH-associated pulmonary vascular remodeling using rhesus macaques as models. In addition, we demonstrate that serial passage of chimeric viruses worsens the pulmonary phenotypes and that differences between SIV nef and SHIV nef may play an important role in the development of severe angioproliferative pulmonary hypertensive disease. Another important contribution of this work is that our findings (summarized in Figure 14) are concordant with features in HIV-PAH, idiopathic PAH, scleroderma PAH, and familial PAH and open the door for future mechanistic studies to elucidate the differences in the nature of PAH-like lesions in the SIV and SHIV nef models.
List of abbreviations

HIV, human immunodeficiency virus
PAH, pulmonary arterial hypertension
HIV-PAH, HIV-associated pulmonary hypertension
SIV, simian immunodeficiency virus
SHIV/\textit{nef}, chimeric simian-human immunodeficiency virus containing HIV \textit{nef}
WPI, weeks post-infection
NEPRC, New England National Primate Research Center
CNPRC, California National Primate Research Center
\(\alpha\)-MHC, alpha myosin heavy chain
\(\beta\)-MHC, beta myosin heavy chain
SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase
ANP, atrial natriuretic peptide
BNP, brain natriuretic peptide
CBC, complete blood counts
IL-12, interleukin-12
GM-CSF, granulocyte macrophage colony stimulating factor-2
sCD40L, soluble CD40 ligand
CCL-2/MCP-1, monocyte chemotactic protein-1
CXCL1/GRO-a, growth-related oncogene alpha
BMP-2, bone morphogenetic protein-2
Mmu, \textit{Macaca mulatta}
IPA, Ingenuity Pathway Analysis
Authors’ contributions

Conception and design of the studies (SCF, NFV, PAL); accountability of all aspects for integrity and accuracy (SA, SCF); acquisition and analyses of clinical endpoints (PAL, SVW), histology (JS, JF, EC), inflammatory biomarkers (LDG), parentage and ancestry verification (SK), cardiac hypertrophy biomarkers (CSL). Bioinformatics predictions and analyses of lung gene expression data (MGE), statistical analyses (SA); wrote the manuscript (SA and SCF); final approval of manuscript (all authors).

Acknowledgements

The authors wish to thank Lourdes Adamson (Center for Comparative Medicine at UC-Davis) for providing expert technical assistance on the virological aspects of the study at CNPRC, Dr. Christopher J. Miller (Department of Pathology, Microbiology & Immunology at UC-Davis) for providing samples from SIV-infected macaques as controls for plasma biomarkers, Dr. Angela Carville (Harvard Medical School) for assistance at NEPRC, and Drs. Andrew D. Miller, Carlyne Cool and Rubin Tuder for histopathological examinations. Finally, the authors wish to extend a posthumous acknowledgement to Dr. Michael Piatak, Jr for his contribution in measuring SIV viral loads.

This study was supported by the NIH/NHLBI grants R01 HL083491, its supplement under the American Recovery and Reinvestment Act, R01 HL059785 (to SCF), T32-HL007171 (UC-Denver Cardiovascular Physiology Laboratory), and NIH/NCATS UL1 TR001082 (Colorado CTSA), R01 AI096966, and R56 AI080418 (to EC). The CNPRC is supported by the NIH Base Operating Grant OD011107. The
NEPRC was supported by NIH/NCRR P51 RR000168, and RR000169. Texas

Biomedical Research Institute is supported by OD011133. Contents are the authors’ sole responsibility and do not necessarily represent official NIH views.
LITERATURE CITED


FIGURE LEGENDS

Figure 1A: History of the *in vivo* passages of the SHIV-*nef* chimeric viruses used to inoculate rhesus macaques. Mandell et al first reported simian AIDS in rhesus macaques (*Macaca mulatta*, Mmu) infected with chimeric SHIV*nef* viruses expressing HIV *nef* in the SIVmac239 genetic backbone. The uncloned virus recovered from their animal Mmu 27747, designated as “SHIV*nef*SF33A” was used to infect their Mmu 28706 and our new cohort of four rhesus macaques housed at the NEPRC (serial passage 1); two uninfected macaques (NEPRC 3 and 6) served as controls (not shown). The uncloned virus recovered from Mmu 28706 was used to infect our cohort of six rhesus macaques at CNPRC (serial passage 2). Each rectangle represents a macaque, whose ID is indicated. Animals were euthanized at the weeks post-infection (WPI) indicated in parentheses. Abbreviations: NEPRC, New England Primate Research Center; CNPRC, California National Primate Research Center.

1B: Plasma SIV viral load in macaques inoculated intravenously with chimeric SHIV-*nef*. Four rhesus macaques were inoculated with SHIV*nef* SF33A at NEPRC. Two uninfected rhesus served as negative controls: one was euthanized after the second experimental macaque was necropsied; the second uninfected control was euthanized after the fourth experimental monkey and monitored from baseline up to 37 weeks PI. Plasma viral load of infected macaques were measured for 29 weeks only. Six rhesus macaques were infected with SHIV*nef* SF33A2 at CNPRC. Each colored line represents data from each macaque, at the indicated weeks post-infection. SIV viral loads are in a logarithmic scale.
Figure 2: Pulmonary vasculature in rhesus macaques infected with SHIV\textit{nef} chimeric virions and parental SIV\text{mac239}. Paraffin-embedded sections from the lungs of rhesus macaques infected with SHIV\textit{nef}_{SF33A} (NEPRC, Panel A), SHIV\textit{nef}_{SF33A2} (CNPRC Panel B), or SIV\text{mac239} (Panel C) were stained with hematoxylin and eosin and examined for vascular pathologies by two independent pathologists. SHIV\textit{nef}-infected monkeys in the NEPRC cohort showed evidence of mild pulmonary vascular pathology (mostly adventitial but no medial thickening) with inflammation (Panel A). In contrast, the CNPRC animals presented evidence of pulmonary vascular remodeling (Panel B). Note that CNPRC 4 showed major remodeling, recanalized obstructive lesion at 37 WPI. In addition, we examined H&E-stained lung sections of two adult rhesus macaques, aged 11 and 14 years old, infected with the parental SIV\text{mac239} for 78-81 weeks (elite controllers). Despite their advanced age relative to our juvenile monkeys, these animals exhibited mild pulmonary vascular pathology (Panel C). Magnifications are shown for each image.

Figure 3: Pentachrome staining in plexiform lesions in the pulmonary vasculature of rhesus macaque CNPRC 4 infected with SHIV\textit{nef}. Paraffin-embedded sections of the lungs of macaque CNPRC 4 were stained with Movat’s pentachrome (Panels A-D). Note that this monkey exhibited major remodeling with obstructive lesions at 37 WPI. Arrows indicate obliterated lumena. Note that the obliterative lesions are composed mostly of fibrin (bright red) and muscle (red) surrounded by mucin (light blue in Panels A-B). Elastic fibers are shown in black (Panels C and D). Collagen/reticular fibers are shown in yellowish color in Panel C. All images were captured at 200X magnification.
Figure 4. Detection of HIV Nef protein in the pulmonary vasculature of a SHIV-nef-infected macaque exhibiting pulmonary vascular lesions after 37 weeks post-infection. Formalin-fixed paraffin-embedded lung tissue of macaque CNPRC 4 was stained with anti-Nef antibody and counterstained with hematoxylin (Panels A-B). The area enclosed by the box in Panel A is shown at higher magnification (indicated by arrow). Magnifications are shown for each image.

Figure 5: Extra-pulmonary vasculature in macaque CNPRC 4 exhibiting significant pulmonary vascular remodeling. Animal CNPRC 4 showed complex obliterative lesions in the pulmonary vasculature at necropsy at 37 weeks post-infection (see Figure 3, panel B). Paraffin-embedded sections of the vasculature in cervical lymph node, kidney, spleen, colon, and liver of this animal were stained with hematoxylin and eosin and examined. No significant vascular remodeling was observed in any of these organs, suggesting that SHIVnef infection leads to pulmonary-specific vascular remodeling. All images were captured at a 200X magnification.

Figure 6. Phenotypic characterization of cells in the pulmonary vasculature of rhesus macaques infected with SHIVnef. Immunofluorescent staining for smooth muscle actin (yellow, Panel A), macrophages (CD163 in yellow, Panel B), proliferative cells (Ki-67 in yellow, Panel C), stem cells (CD34 in yellow, Panel D), and T lymphocytes (CD3 in yellow, Panel E). Each staining was multiplexed with staining for endothelial cells (Von Willebrand factor, green), Nef (red) and nuclei (blue). Macaques
with statistically significant higher immunofluorescence are shown (see Figure 7 for quantification results); macaque ID and weeks post infection (WPI) are shown on top of each panel. The insets indicated by rectangles on the left are shown to the right, in higher magnification. Panel F shows the isotype controls (mouse IgG, yellow) multiplexed with staining for endothelial cells and nuclei. All figures show merged images. Magnifications are shown for each figure.

Figure 7. Quantification of immunofluorescent signals in the pulmonary vasculature of rhesus macaques infected with SHIV_{nef}. Immunofluorescent signals for HIV Nef (Panel A), von Willebrand factor for endothelial cells (Panel B), CD163 for macrophages (Panel C), CD3 for lymphocytes (Panel D), SMA for smooth muscle actin (Panel E), CD34 for stem cells (Panel F), and Ki-67 for proliferation (Panel G) were quantified in Image J and analyzed statistically. The adjusted p-values from the Kruskal-Wallis test followed by Dunn’s multiple comparison tests are shown for the significant quantifications. Abbreviations: MFI, mean fluorescence intensity.

Figure 8. Immunohistochemical analysis of microthrombi in the pulmonary vasculature of SHIV_{nef}-infected rhesus macaques. Intravascular staining of the platelet marker CD61, microthrombi. CD61 sections was detected by immunoperoxidase (brown) and counterstained with hematoxylin. Note the staining pattern in CNPRC 4, suggestive of intravascular platelet aggregates. Magnifications are shown for each image.
Figure 9. Cardiac hypertrophic gene expression in SHIV nef-infected macaques. Total RNA from the right and left ventricle was extracted and analyzed by real time PCR. We found that genetic expression of ANP, α-MHC, and β2 adrenergic receptors were evidently increased in the right ventricle of macaque CNPRC 4. In addition, left ventricular gene expression in macaque CNPRC 4 switched from α and β isoform of MHC. Ventricular BNP was also increased in CNPRC 4 and 5. The relative abundance of SERCA was decreased in SHIV nef macaques relative to the control. All these changes in gene expression are associated with ventricular dysfunction and hypertrophy. Abbreviations: SERCA, sarco(endo)plasmic reticulum Ca²⁺ ATPase; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

Figure 10. Hematologic abnormalities in SHIV- nef infected macaques. Longitudinal complete blood counts (CBC) were measured in SHIV nef-infected macaques at NEPRC and CNPRC and analyzed relative to normal CBC values in male Rhesus macaques (Gill 2012 and Chen 2009); normal reference intervals are shaded in gray. Hematologic parameters are shown in the Y-axes; macaque IDs are shown in the X-axes. Each symbol represents independent data points collected for each macaque; the horizontal line indicates the median value. Blood cell abnormalities were defined if the median CBC values exceeded or were below the normal reference values (gray shaded areas).

Figure 11. Comparison of CBC values between SHIV- nef infected macaques in NEPRC and CNPRC. Complete blood counts (CBC) were performed throughout the course of the infection with SHIV nef infected macaques (4 NEPRC and 6 CNPRC animals). Each hematologic parameter is shown in the Y-axis; each macaque group is
indicated on the X-axis. Data are represented by scatter dot plots with lines at the mean with SEM. White blood cells and lymphocyte data were analyzed by non-parametric Mann-Whitney U tests; Student’s T tests were used to analyze the other parameters. P values are shown for each parameter analyzed.

**Figure 12. Detection of inflammatory cytokines in plasma of rhesus macaques infected with SHIV\textit{nef} and SIVmac\textit{239}**. Plasma cytokines were measured by Luminex at different times during the course of lentiviral infection. The figure includes the cytokines that were statistically different between the two macaque groups. The lines indicate mean with SEM.

**Figure 13. Inflammatory upstream regulators in the lungs of SHIV\textit{nef}-infected rhesus macaques**. Gene expression from 84 inflammatory genes was measured in the lungs of infected rhesus macaques and their direction of change used to identify activated or inhibited upstream regulators affecting the system. BMP-2 (measured directly) and RelB (not measured on the array) were predicted to be the most inhibited (z-score = -2.6) and activated (z-score = 2.8) molecules respectively due to SHIV\textit{nef} infection by the IPA software. Targets known to be induced by BMP-2 are all shown to be down (green) in the array, while genes known to either be induced or inhibited by RelB are up (red/pink) and down (green) respectively. The only downstream target not reflective a more active RelB is IL1B (yellow line indicates inconsistent relationship). Dashed lines indicate indirect and solid lines indicate direct biological relationships between the connected genes, while blue (inhibited) and orange (activated) lines signify
the activation state of the effector molecule based on the expression of the connected target.

**Figure 14. Summary of differences molecular and histopathological findings between SHIV\textit{nef}- and SIVmac239-infected macaques and concordance with different forms of PAH.** The most relevant findings of our study are listed. Most of our observations in SHIV\textit{nef}-infected macaques are consistent with several forms of PAH (iPAH colored in pink, Ssc-PAH in blue, and f-PAH in green). Abbreviations: iPAH, idiopathic pulmonary arterial hypertension; fPAH, familial PAH; SSc-PAH, systemic sclerosis-associated PAH.
Table 1. Hematologic abnormalities in SHIV- nef infected macaques. Longitudinal complete blood count (CBC) in SHIV nef-infected macaques at NEPRC (top panel) and CNPRC (bottom panel). Macaque IDs are shown, with the times post-infection in parenthesis. CBC data were analyzed relative to normal CBC values in male Rhesus macaques (Gill 2012 and Chen 2009). Values were categorized as blood cell abnormalities if the median values exceeded the normal intervals (up arrows) or were below the normal intervals (down arrows). Median values within the normal intervals are shown as horizontal arrows. Data were analyzed by one-way ANOVA with Dunn’s multiple comparison tests to compare CBC values of each macaque with the rest of his mates. P values were determined with Gaussian approximations and are shown only for statistically significant CBC values. Statistical significance was set at p < 0.05.

Table 2. Comparison of CBC values between SHIV- nef infected macaques in NEPRC and CNPRC. Complete blood counts (CBC) were performed throughout the course of the infection with SHIV nef-infected macaques. White blood cells and lymphocyte data were analyzed by non-parametric Mann-Whitney U tests; Student’s T tests were used to analyze the other parameters. P values are shown for each parameter analyzed.

Table 3. Plasma cytokines levels in six CNPRC SHIV nef-infected macaques relative to four SIV-infected monkeys. Table shows the linear mixed effects model estimates for group-specific intercepts and slopes for weeks post-infection for each cytokine.
Table 4. Inflammatory gene expression in SHIV nef- compared to SIV-infected rhesus macaques. Lung RNA samples were amplified using the PCR targeted array for Rhesus Macaque Inflammatory Cytokines & Receptors (SABiosciences) in a BioRad iCycler iQ, as per manufacturer protocols. The housekeeping genes beta-2-microglobulin and hypoxanthine-guanine phosphoribosyltransferase-like were used for normalization. Gene expression was determined via the ΔΔCt method; fold changes were calculated based on difference in gene expression between six SHIV nef- and two SIVmac239-infected macaques. Statistical analyses were not performed given the small sample size in the SIV group. Only the genes with altered expression of genes at fold change cutoff of ± 2.7 are shown. Abbreviation: Mmu, Macaca mulatta
Figure 1A

Mandell 1999 SHIV-nef cohort

SHIV_{nef}^{SF33A2}

Mmu 27747
(53 WPI)

SHIV_{nef}^{SF33A}

Mmu 28706
(65 WPI)

New SHIV-nef cohort (NEPRC)

- NEPRC 1
  - (12 WPI)
- NEPRC 2
  - (16 WPI)
- NEPRC 4
  - (27 WPI)
- NEPRC 5
  - (80 WPI)

New SHIV-nef cohort (CNPRC)

- CNPRC 1
  - (12 WPI)
- CNPRC 2
  - (13 WPI)
- CNPRC 3
  - (24 WPI)
- CNPRC 4
  - (37 WPI)
- CNPRC 5
  - (52 WPI)
- CNPRC 6
  - (62 WPI)

Figure 1B

NEPRC cohort
Figure 2

|--------------------------------- SHIV nef (SIVmac239 backbone) ---------------------------------|  |  |
|--------------------------------- SHIV nef (SIVmac239 backbone) ---------------------------------|  |  |

A

NEPRC 1, 12 WPI
400X

NEPRC 2, 16 WPI
200X

NEPRC 3, negative
400X

NEPRC 4, 27 WPI
400X

B

CNPRC 1, 12 WPI
200X

CNPRC 2, 13 WPI
400X

CNPRC 3, 24 WPI
400X

CNPRC 4, 37 WPI
200X

CNPRC 5, 50 WPI
200X

CNPRC 6, 62 WPI
200X

C

SIV\(_{mac239}\) #1
200X

SIV\(_{mac239}\) #2
200X

400X
Figure 4
Figure 6

A. Smooth muscle actin in CNPRC 4 at 37 WPI

B. Macrophages in CNPRC 2 at 13 WPI

C. Proliferative cells in CNPRC 4 at 37 WPI

D. Hematopoietic stem cells in CNPRC 4 at 37 WPI

E. T lymphocytes in CNPRC 1 at 12 WPI

F. Isotype controls
Figure 7

A

B

C

D

E

F

G
Figure 11

- White Blood Cells (x1,000 cells/μl): p = 0.1434

- Hemoglobin (g/dL): p < 0.0001

- Red Blood Cells (x 10^6 cells/μl): p < 0.0001

- Hematocrit (%): p < 0.0005

- Lymphocytes (cells/μl): p = 0.0046

- Platelets (x 10^5 cells/μl): p = 0.8917
Figure 14

SHIVnef vs. SIV\textsubscript{mac239}

- Cardiac hypertrophy
- Increased IL-2 and GM-CSF
- Changes in CCL2
- Decreased BMP-2
- Pulmonary plexiform lesions (muscle/fibrin-rich)
- Increased CCL23

f-PAH

IPAH

SSc-PAH
## Table 1

### NEPRC

<table>
<thead>
<tr>
<th></th>
<th>NEPRC 1 (12 WPI)</th>
<th>NEPRC 2 (16 WPI)</th>
<th>NEPRC 4 (27 WPI)</th>
<th>NEPRC 5 (80 WPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Platelets</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
</tbody>
</table>

### CNPRC

<table>
<thead>
<tr>
<th></th>
<th>CNPRC 1 (12 WPI)</th>
<th>CNPRC 2 (13 WPI)</th>
<th>CNPRC 3 (24 WPI)</th>
<th>CNPRC 4 (37 WPI)</th>
<th>CNPRC 5 (50 WPI)</th>
<th>CNPRC 6 (62 WPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Platelets</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Mean cell hemoglobin</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Monocytes</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
<td>⇐</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>CBC parameter</th>
<th>NEPRC</th>
<th>CNPRC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>mean</td>
<td>95% CI</td>
<td>median</td>
</tr>
<tr>
<td>White blood cells (x 1000/μl)</td>
<td>7.46</td>
<td>7.92</td>
<td>6.87, 8.71</td>
<td>8.10</td>
</tr>
<tr>
<td>Red blood cells (x 10⁶ cells/μl)</td>
<td>5.93</td>
<td>5.91</td>
<td>5.80, 6.03</td>
<td>5.05</td>
</tr>
<tr>
<td>Lymphocytes (cells/μl)</td>
<td>2180</td>
<td>2598</td>
<td>2130,3067</td>
<td>3120</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.10</td>
<td>13.18</td>
<td>12.90,13.47</td>
<td>11.60</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39.8</td>
<td>39.7</td>
<td>38.5, 40.8</td>
<td>35.1</td>
</tr>
<tr>
<td>Platelets (x 10⁵ cells/μl)</td>
<td>2.97</td>
<td>2.98</td>
<td>2.77, 3.19</td>
<td>2.82</td>
</tr>
<tr>
<td>Cytokine</td>
<td>SIV-infected</td>
<td>SHIV-nef infected</td>
<td>p-value</td>
<td>SIV-infected</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>------------------</td>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>CXCL1</td>
<td>2.90</td>
<td>2.2003</td>
<td>0.0038</td>
<td>0.02</td>
</tr>
<tr>
<td>INFγ</td>
<td>2.37</td>
<td>1.6011</td>
<td>0.0566</td>
<td>-0.02</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.56</td>
<td>1.6472</td>
<td>0.0002</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-15</td>
<td>2.47</td>
<td>2.2165</td>
<td>0.4359</td>
<td>-0.01</td>
</tr>
<tr>
<td>IL1-Ra</td>
<td>2.57</td>
<td>2.4728</td>
<td>0.5305</td>
<td>0.00</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.33</td>
<td>2.1582</td>
<td>0.2886</td>
<td>0.01</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.60</td>
<td>0.7747</td>
<td>0.7988</td>
<td>-0.00</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>-0.16</td>
<td>0.6079</td>
<td>&lt;0.0001</td>
<td>0.00</td>
</tr>
<tr>
<td>IFNα</td>
<td>1.70</td>
<td>1.2695</td>
<td>0.3438</td>
<td>-0.01</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.20</td>
<td>1.5184</td>
<td>0.3112</td>
<td>-0.04</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.39</td>
<td>1.7642</td>
<td>0.4177</td>
<td>-0.00</td>
</tr>
<tr>
<td>IP-10</td>
<td>3.42</td>
<td>3.0348</td>
<td>0.1993</td>
<td>0.01</td>
</tr>
<tr>
<td>CCL2</td>
<td>2.42</td>
<td>2.1334</td>
<td>0.0063</td>
<td>0.00</td>
</tr>
<tr>
<td>sCD40L</td>
<td>2.34</td>
<td>1.1867</td>
<td>0.0002</td>
<td>0.00</td>
</tr>
<tr>
<td>Gene</td>
<td>Gene symbol</td>
<td>Fold change in SHIV nef infection</td>
<td>Cellular function/pathway</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------------</td>
<td>-----------------------------------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>Interleukin 12 receptor beta 1 subunit</td>
<td>IL-12RB1</td>
<td>-6.7</td>
<td>JAK-STAT signaling pathway</td>
<td></td>
</tr>
<tr>
<td>CD40 ligand</td>
<td>CD40L</td>
<td>-6.4</td>
<td>Immunoglobulin class switch, adaptive immunity</td>
<td></td>
</tr>
<tr>
<td>TNF (ligand) superfamily, member 14</td>
<td>TNFSF14</td>
<td>-5.7</td>
<td>NF-kB signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Lymphotoxin alpha</td>
<td>LTA</td>
<td>-4.8</td>
<td>Inflammatory, immunostimulatory, and antiviral responses, apoptosis</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C) ligand 17</td>
<td>CCL17</td>
<td>-4.5</td>
<td>Antimicrobial, trafficking and activation of mature T cells</td>
<td></td>
</tr>
<tr>
<td>Interleukin 3</td>
<td>IL-3</td>
<td>-4.3</td>
<td>Cell growth, differentiation and apoptosis</td>
<td></td>
</tr>
<tr>
<td>Interleukin 1 receptor type 1</td>
<td>IL1R1</td>
<td>-4.1</td>
<td>Interleukin 1 receptor antagonist, MAPK signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Fractalkine</td>
<td>CX3CL1</td>
<td>-4.0</td>
<td>T lymphocyte and monocyte chemoattractor</td>
<td></td>
</tr>
<tr>
<td>Interleukin 13 receptor subunit alpha-2-like</td>
<td>LOC708339</td>
<td>-3.4</td>
<td>JAK-STAT signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Interleukin 13</td>
<td>IL-13</td>
<td>-3.3</td>
<td>B-cell maturation and differentiation</td>
<td></td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>TNFRSF11B</td>
<td>-3.0</td>
<td>Blocks the binding of TNF-related apoptosis-inducing ligand (TRAIL)</td>
<td></td>
</tr>
<tr>
<td>CD70</td>
<td>CD70</td>
<td>-3.0</td>
<td>TNF ligand family, regulates B-cell activation</td>
<td></td>
</tr>
<tr>
<td>Fas ligand</td>
<td>Fas L</td>
<td>-2.8</td>
<td>TNF superfamily, apoptosis</td>
<td></td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>IL-6</td>
<td>2.8</td>
<td>Multifunctional cytokine, inflammatory response</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C) receptor 8</td>
<td>CCR8</td>
<td>2.9</td>
<td>G-protein coupled receptor signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Interferon-inducible T-cell alpha chemotactant</td>
<td>CXCL11</td>
<td>3.0</td>
<td>Chemotactic for activated T cells, CXCR3 signaling pathway</td>
<td></td>
</tr>
<tr>
<td>TNF (ligand) superfamily, member 13b</td>
<td>TNFSF13B</td>
<td>3.0</td>
<td>NF-kB signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Monocyte chemotactic protein 1</td>
<td>CCL2</td>
<td>3.7</td>
<td>Recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation</td>
<td></td>
</tr>
<tr>
<td>Macrophage Inflammatory Protein 3a</td>
<td>CCL20</td>
<td>3.8</td>
<td>Strongly chemotactic for lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Monocyte chemotactic protein 3-like</td>
<td>LOC714751</td>
<td>4.1</td>
<td>Attracts macrophages during inflammation</td>
<td></td>
</tr>
<tr>
<td>Interleukin 1 receptor, type 2</td>
<td>IL-1R2</td>
<td>5.0</td>
<td>MAPK signaling pathway</td>
<td></td>
</tr>
<tr>
<td>Macrophage Inflammatory Protein 3</td>
<td>CCL23</td>
<td>11.6</td>
<td>Highly chemotactic for resting T cells and monocytes</td>
<td></td>
</tr>
</tbody>
</table>